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Lentiviral Vectors

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Biosafety Issues in Lentivector Production

C. Delenda, M. Audit, and O. Danos

1 Introduction

The development of efficient lentivectors brings about exciting possibilities for novel therapeutic interventions. Still, as new biologicals intended to be used in the clinic, these vectors will have to comply with a complete set of requirements regarding their mode of preparation and characterization. Over the past 50 years, there has been an increasing awareness of the safety issues surrounding the manufacturing of medicinal products. Regulatory authorities and agencies regularly publish guidelines and coordinate international conferences on harmonization (ICH), whose goal is to define common standards for biotechnological and biological products to be administered to human patients (Schultz 1998).

Genethon III, 1 bis rue de l'Internationale, 91002 Evry Cedex 02, France

Table 1. Cellular proteins encapsidated into retroviruses

Proteins	Interaction	Virus	References
Cyclophilin A	CA region of *gag*	HIV-1	(Braaten et al. 1996)
MHC class I and II Adhesion molecules: LFA-1, CD43, CD44, CD55, CD59, CD63, CD71, CR3 (CD11b), CR4 (CD11c), ICAM-1/2 Adhesion molecules: LFA-1, ICAM-1, HLA-DR, HLA-DQ, HLA-DP, β2-microglobulin HO-3 (homology to histidyl aminoacyl-tRNA synthetase) EF1α Cytoskeletal proteins: actin, ezrin, moesin, cofilin	Surface of virions, heparan binding Surface of virions Surface of virions Surface of virions MA region of *gag* MA region of *gag* Inside virions, prior to protease cleavage	HIV-1 (Group M), SIVCPZ HIV-1 (wt, VSV-G or MLV pseudotyped) HIV-1 HIV-1 (laboratory strains) HIV-1 (primary isolates) HIV-1 (wt, VSV-G pseudotyped) HIV-1 HIV-1	(Aiken 1997; Saphire et al. 1999) (Arthur et al. 1992) (Castiletti et al. 1995) (Saifuddin et al. 1995) (Frank et al. 1996) (Capobianchi et al. 1994) (Cantin et al. 1996) (Lama and Trono 1998) (Ciminarelli and Luban 1999) (Ott et al. 1996; Rey et al. 1996)
Ubiquitin	*Gag* p6 protein *Gag* p12 protein *vpu* and *gag*	HIV-1, SIV Mo-MuLV HIV-1	(Ott et al. 1998) (Callahan et al. 1998)
UBP (*vpu*-binding protein, member of the tetratricopeptide repeat family)			
ERK/MAPK (mitogen-activated protein kinase)	Inside virions, MA phosphorylation	HIV-1	(Jacque et al. 1998)
HSlau (human Staufen, double-stranded RNA-binding protein)	Genomic RNAs	HIV-1, HIV-2, Mo-MuLV	(Mouland et al. 2000)

against the vector particles and the transgene product) will have to be tested in animals. The data available on in vivo studies with lentivectors indicate that there is no major nor reproducible toxicity of the preparations. When observed several weeks or months following injection, muscle, brain, liver, eye and cochlea of rodents express the transgene and do not contain noticeable lymphocyte or macrophages infiltrate (Gallichan et al. 1998; Han et al. 1999; Kafri et al. 1997; Takahashi et al. 1999; Zufferey et al. 1998). Early examination of the injection site has documented the presence of inflammatory cells (Dull et al. 1998; Naldini et al. 1996a,b), but since those were also observed in control animals, they were attributed to the surgical procedures (Blomer et al. 1997; Miyoshi et al. 1997). Lentivector gene transfer into the monkey nigrostriatal system has been shown to induce minor perivascular cuffing without apparent inflammatory response (Kordower et al. 1999). Studies on the liver describe a dose-dependent increase in serum ALT after intraportal infusion of lentivector in Fischer rats, and a mortality rate of 74% at a dose of 8×10^8TU (Park et al. 2000). Such observations point to a potential toxic effect, likely due to contaminants. The presence of contaminants triggering a local inflammatory response shortly after injection into a tissue can dramatically influence the onset of an immune response against the transgene product. The control over these initial events involving innate immunity depends on the quality of the vector production and purification processes.

3 Improving the Biosafety of Lentivectors

3.1 Design of Vector Systems

Most lentivector production systems involve splitting the viral genome into individual helper plasmid constructs. Dividing the different viral elements into separate counterparts diminishes the risk of productive recombination with the vector genome. These plasmids are transfected into cell lines and lentivectors are then produced in the culture supernatant over a few days. The following sections describe how three (or more) generations of designs have improved the biosafety of lentivectors, as it relates to the different items discussed above.

3.1.1 First Generation Vectors

First generation LV vectors (Fig. 1A) are produced by co-transfecting into cultured cells three plasmid constructs which contain the viral genes as well as the minimal *cis*-acting sequences necessary for the formation of vector particles. The first plasmid encodes the Gag and Pol viral core proteins. It is derived from the original viral genome by deleting the env gene and replacing the viral LTR sequences by nonretroviral promoter and polyadenylation sequences. As in classical retroviral vectors, the packaging signal (ψ) has been removed in order to reduce encapsida-

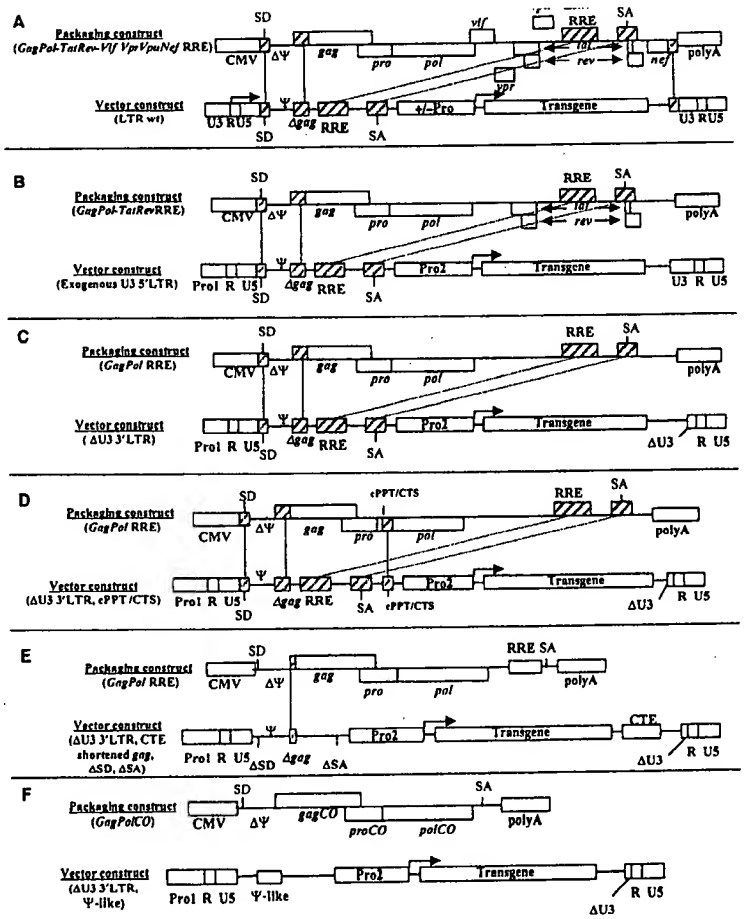

Fig. 1A–F. Regions of homology between packaging and vector constructs. Schematic representations of the different structures of LV constructs used in lentivector production. First (A), second (B) and third (C–F) vector generations are shown. In addition, this diagram highlights tat-dependent (A–B) or tat-independent (C–F) vectors, as well as rev-dependent (A–D) and rev-independent (E–F) vectors. A fourth plasmid encoding rev protein is needed in C and D. Regions of homology between packaging and vector constructs are depicted as *hatched brackets* and the different genetic elements are shown as *opened brackets*. *CMV*, human cytomegalovirus immediate-early promoter; *Pro*, heterologous promoter; *Pro1* and *Pro2* are referred for promoters initiating LV primary and internal transgene transcriptions, respectively. CMV and Rous sarcoma virus (RSV) promoters are the most commonly used for Pro1; *Δ*, deletions; *SD*, splice donor site; *SA*, splice acceptor site; *ψ*, encapsidation signal (stem loops SL1 to 3); *Δgag*, truncation of gag (SL4); *RRE*, rev responsive element; *cPPT/CTS*, central polypurine tract associated with the central terminating sequence; *gagCO*, *proCO*, *polCO*, helper LV codon-optimized (*CO*) genes

tion of the virus-like transcripts encoding Gag and Pol. The second plasmid provides information for an heterologous envelope glycoprotein, allowing the extention of the restricted lentiviral host cell tropism. Most commonly, the VSV G glycoprotein is used, which yields high vector titer and confers greater stability to the vector particles. Other viral envelope glycoproteins include amphotropic and 10A1 MLV, gibbon ape leukemia virus (GALV) (Page et al. 1990; Stitz et al. 2000) and the rabies G protein (Reiser 2000). Finally, the third construct is designed to produce the transfer vector RNA which is encapsidated into the pseudotyped particles. It contains all sequences needed for the production and packaging of active lentivector genomes (LTR, ψ, RRE) and the expression cassette for the transgene of interest.

It is important to note that according to this design, more than 65% of the original genetic information of the virus is absent and would have to be accidentally complemented by exogenous components in order to generate a replication-competent structure.

3.1.2 Second Generation Vectors

Several lentiviral genes (*vif*, *vpr*, *vpu* and *nef*) are not essential for viral replication in vitro, but crucially important for viral pathogenesis in vivo. Their presence in vectors may raise safety concerns because the proteins they encode have cytotoxic activities. For instance, Vpr induces G2 cell cycle arrest and apoptosis (Bukrinsky and Adzhubei 1999) and Nef alters the cellular activation pathways (Hanna et al. 1998). Cell surface molecules such as CD4 and the class I major histocompatibility complex are down-regulated by Nef and Vpu (Piguet et al. 1999). Nef, Vpr and Vif are incorporated into the viral particles and could enhance the immunogenicity of vectors. It has therefore been important to demonstrate that lentivectors could be efficiently produced in the absence of these nonessential genes (Fig. 1B). These second generation vectors are reportedly equally efficient for transduction both in vitro and in vivo (Zufferey et al. 1997). Yet, evidence has been found that *vif* and *vpu* may be required for optimal transduction of the liver (Kafri et al. 1997) and of resting lymphocytes (Chinnasamy et al. 2000).

3.1.3 Third Generation Vectors

Recent studies indicate that the transactivator Tat is also dispensable for generation of fully efficient lentivectors. In this design (Fig. 1B), the Tat-dependent 5′ LTR classically used for generating the vector genomic RNA is replaced by strong heterologous promoter sequences from the human cytomegalovirus immediate-early promoter or the Rous sarcoma virus U3 sequence (Dull et al. 1998; Kim et al. 1998; Miyoshi et al. 1998). An additional improvement in safety is brought by further splitting the original viral genome and expressing Rev from a fourth separate construct. The third generation vectors display only marginal (two- to threefold) reduction in transduction efficiency.

The self-inactivating (SIN) design described for MLV vectors (Cone et al. 1987) has been successfully adapted to lentivectors. It involves the deletion of the

U3 region in the 3′ LTR of the transfer vector, removing most of the viral transcriptional control elements which would be active in the integrated proviral state (Iwakuma et al. 1999; Mangeot et al. 2000; Miyoshi et al. 1998; Schnell et al. 2000; Zufferey et al. 1998). This modification prevents transcriptional interference between the LTR and the internal promoter and allows for a better control over transgene expression. Crippling the LTR also reduces the odds of RCR generation, and may prevent transcriptional activation of cellular genes adjacent to the provirus. Although this feature is usually advertised as a prime advantage of SIN vectors, it should be noted that the presence of an active promoter internal to the construct is associated with equivalent risks of transcriptional read-through. A better way of tempering read-through, which is mostly due to a deficient cleavage and polyadenylation of vector transcripts within the 3′ LTR (Swain and Coffin 1992; Zhang et al. 1998), is to replace the retroviral polyadenylation signal by exogenous ones (e.g., β-globin or SV40). In this design, vector safety is improved also because additional viral sequences are removed from the vector and unexpectedly, it results in increased titers (Iwakuma et al. 1999). Altogether, this further crippling of the original helper genome and modification of the transfer vector result in vector systems where up to 80% of the initial LV sequences have been removed, without notable changes in gene transfer performances.

3.1.4 Additional Improvements

The risk of recombination can be further reduced by splitting the packaging construct into two separate counterparts, one expressing *gag-pro* and the other expressing *pol*. Vectors have been successfully produced using this approach (Wu et al. 2000). Until recently, optimal vector production has required the presence of rev, which interacts with the RRE sequence and positively affects the nuclear export of both the unspliced *gag-pol* mRNAs and the transfer vector genomic RNA. Several solutions have now been proposed for the design of rev-independent production systems. The RRE sequences can be replaced by heterologous viral sequences known to enhance export and/or stability of unspliced transcripts. These are the constitutive transport element (CTE) from the Mason-Pfizer virus (Gasmi et al. 1999; Srinivasakumar and Schuening 1999) or from the simian retrovirus type 1 (Mautino et al. 2000a), or the post-transcriptional regulatory elements from human or woodchuck hepatitis B viruses (HPRE and WPRE, respectively) (Zufferey et al. 1999). In the absence of Rev, the *gag* and *gag-pol* mRNAs are targeted for degradation through inhibitory sequences (NIS) present in the coding region. Codon optimization of the *gag-pol* gene has led to the inactivation of NIS and to an enhanced protein production (Kotsopoulou et al. 2000; Wagner et al. 2000). It also reduces the sequence homology between the packaging construct and adventitious partners for recombination. Finally, removing the donor splice site in the transfer vector has yielded higher titers and enhanced levels of unspliced cytoplasmic mRNAs in the absence of *rev* (Mautino et al. 2000b).

The polypurine tract, located in a central position of the lentiviral genomes (cPPT), facilitates nuclear translocation of the pre-integration complex and has been shown to enhance HIV-1 vector efficiency into both dividing and nondividin cells (Follenzi et al. 2000; Zennou et al. 2000). From a safety point of view, or minor drawback for the use of the cPPT sequence is that it adds sequences prese in the pol gene of the helper construct to the vector and therefore increases th chances of recombinations. Considering the improvement in vector efficienc which is consistently around one order of magnitude, and the fact that all of th above mentioned safety features are still present in the production system, th addition of the cPPT remains a benefit.

Further deletions or substitutions in the vector genome can be envisioned. A in vitro-selected RNA with high affinity for the HIV-1 nucleocapsid (NC) protei has been shown to mediate packaging into HIV-1 virions and could be substitute to the viral sequence (Berglund et al. 1997; Clever et al. 2000). In addition, should be possible to reduce the length of the LTR region as HIV-1 mutants wit deletions in the 3′ R sequences still replicate efficiently (Berkhout et al. 1995 Finally, studies with oncoretroviruses have shown that the Ψ packaging sequence i efficiently removed from the vector genome during reverse transcription whe placed between direct repeats (Delviks et al. 1997). Extending this to lentivector would enhance safety by preventing vector mobilization and spread, and RCI generation involving Ψ sequence rescue.

3.2 Stable Packaging Cell Lines

Even though the transient transfection procedure allows for efficient and saf lentivector production, the generation of clinically acceptable vectors will ulti mately require stable producer cell lines. This will eliminate the risk of homologou recombination between the transfected plasmids, as well as the problem of carryin over plasmid DNA in the vector batches. Chiefly, it will facilitate the standar dization and scaling-up of vector production.

Several groups have described the isolation and characterization of cell line producing vectors with either the original Env proteins (Kaul et al. 1998; Yu et al 1996) or VSV-G-pseudotyped (Kafri et al. 1999; Klages et al. 2000). The mos advanced cell lines have been built using the design of third generation vectors (se above) (Klages et al. 2000). The fact that the lentiviral protease, Rev and the VSV G proteins are cytotoxic or cytostatic when constitutively expressed (Kaplan an Swanstrom 1991; Rolls et al. 1994) mandates the use of an inducible system Tetracycline induced silencing of the transfected constructs has been preferred because it allows the production of lentivectors in the absence of drugs, a preferre situation for manufacturing clinical grade material.

3.3 Lentivector Production Using Viral Shuttles

Another strategy to produce retroviral vectors relies on the use of powerfu expression systems derived from herpes simplex virus (HSV), Semliki Forest viru